AD_____

Award Number: DAMD17-00-1-0551

TITLE: Role of CD44 in Malignant Peripheral Nerve Sheath Tumor
Growth and Metastasis

PRINCIPAL INVESTIGATOR: Larry S. Sherman, Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45267-0553

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2001	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Role of CD44 in Malignant Peripheral Nerve Sheath Tumor Growth and Metastasis

5. FUNDING NUMBERS
DAMD17-00-1-0551

6. AUTHOR(S)
Larry S. Sherman, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Cincinnati
Cincinnati, Ohio 45267-0553

E-Mail: Lawrence.Sherman@uc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Malignant peripheral nerve sheath tumors (MPNST) are aggressive, difficult to treat tumors that occur in type I neurofibromatosis patients with an increased incidence compared to the general population. These tumors metastasize to a number of sites, including the lungs, and have a poor 5 year survival rate. We previously found that MPNSTs overexpress the CD44 tranmembrane glycoprotein and that reducing CD44 expression inhibits MPNST cell invasion. We also found that aberrant CD44 expression is linked to overexpression of the epidermal growth factor receptor (EGFR) in these cells through a Ras-independent mechanism. Here, we provide evidence that EGFR upregulates CD44 expression in the ST8814 cell line through a mechanism that depends on Src kinase. Furthermore, we show that MPNST cell invasion depends on an autocrine loop involving HGF, an HGF activating enzyme (HGFA), and c-Met, all of which are expressed by MPNST cells. We are currently conducting studies that link CD44 to increased c-Met activity and signaling, and determining the mechanism by which CD44 influences the HGF-c-Met autocrine loop. These studies provide new insights into the mechanisms of MPNST invasion as well as potential targets for the treatment of these tumors.

14. SUBJECT TERMS
Neurofibromatosis, Malignant Peripheral Nerve Sheath Tumor, CD44, c-Met, invasion

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Annual Progress Report
Role of CD44 in Malignant Peripheral Nerve Sheath Tumor Growth and Metastasis
DAMD17-00-1-0551

September 22, 2001

INTRODUCTION

Malignant peripheral nerve sheath tumors (MPNST) are aggressive, difficult to treat tumors that arise within peripheral nerves. Although extremely rare in the general population, MPNST occur with significantly higher frequency (approximately 5%) in patients with type I neurofibromatosis (NF1) and contribute significantly to the morbidity and mortality of affected patients. This study is founded on the belief that by discovering the molecular mechanisms underlying MPNST invasion and metastasis we will gain clues about how to treat these tumors. We are focusing on the role of the CD44 transmembrane glycoprotein in this process, as we have previously found that CD44 proteins are overexpressed by MPNST (Sherman et al., 1997) and since these proteins have been implicated in tumor growth and metastasis (reviewed by Naor et al., 1997).

BODY

Although we have made substantial progress that is consistent with our approved Statement of Work (outlined below), the initiation of these studies was delayed due to changes in personnel. Dr. Rachel Bolante, who was considering joining our group to work on this project, decided to join another lab at about the time the funding for this project became available (September 1, 2000). As a result, we hired another postdoctoral fellow, Dr. Weiping Su (who recently obtained a Ph.D. from the University of Tokyo Department of Veterinary Pathology). However, due to unexpected delays in the processing of Dr. Su's visa application, she was unable to start working until March 1, 2001. This report, therefore, describes findings and achievements of only the past 7 months. We wish to request a 6 month extension on this project, so that we will have a full 2 years to achieve the proposed goals.

Objective 1: Determine if epidermal growth factor receptor (EGFR)-dependent Src signaling influences invasion and CD44 expression in MPNST cells.

The initial goal of this aim is to determine if Src kinase activity is elevated in MPNST cells *in vitro*. Although we have some preliminary data consistent with elevated Src activity, we are still optimizing this assay in our lab, and should have results from these experiments in the next 2-3 months. We have, however, made substantial progress using Src inhibitors (PP1, PP2 and CGP77675) to inhibit CD44 expression by MPNST cells. ST8814 MPNST cells treated with any of these drugs express dramatically reduced levels of CD44 proteins by Western blotting compared to cells treated with vehicle alone (Fig. 1a). Similarly, transient transfection of a dominant negative Src construct in ST8814 cells inhibits CD44 expression (Fig. 1a). We are presently testing the effects of these reagents on MPNST motility and invasion, using a quantitative invasion assay as previously described (Lamb et al., 1997) and as outlined in the original proposal.
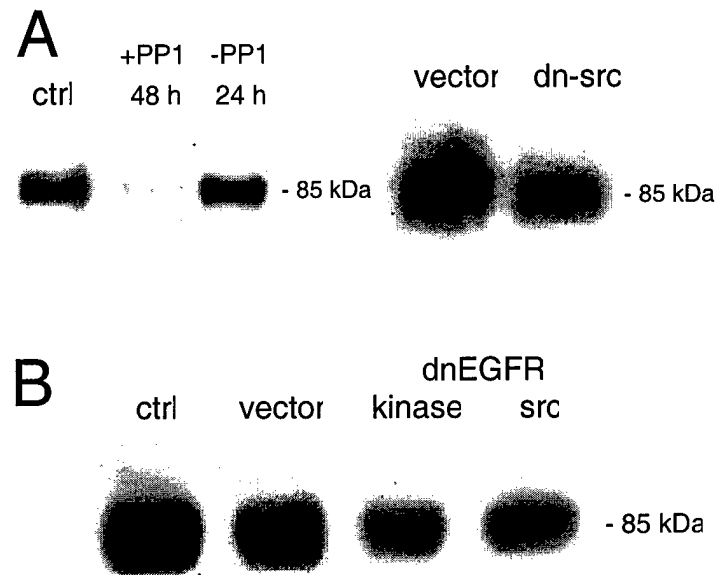
A

+PP1 -PP1
ctrl 48 h 24 h   vector   dn-src

- 85 kDa   - 85 kDa

B   dnEGFR
ctrl   vector   kinase   src

- 85 kDa

Figure 1: Inhibition of EGFR and Src kinase inhibit CD44 expression in MPNST cells. (A, left panel) ST8814 cells were grown in the presence and absence of either the Src inhibitor PP1 (or PP2 or CGP77675, not shown) for 48 hrs. then grown for an additional 24 hrs. in the absence of drug. CD44 expression was then analyzed by western blotting. Equal loading was verified by Ponceau S staining (not shown). Note that inhibiting Src activity reversibly reduces CD44 expression. (A, right panel) ST8814 cells were transiently transfected with a dominant negative (dn) Src construct for 48 hrs. then analyzed for CD44 expression by Western blotting as above. Note that compared to cells transfected with vector alone, cultures transfected with dn-src demonstrated reduced levels of CD44. (B) ST8814 cells were transiently transfected with either a dominant negative EGFR construct lacking the entire kinase domain ("kinase") or the Src association domain ("src") then assayed for CD44 expression as above. Note that both dominant negative constructs resulted in reduced CD44 expression compared to untransfected control cultures ("ctrl") and cultures transfected with vector alone. Transfection efficiency in these assays is trypically less than 25%. We are presently repeating these assays with co-transfection of pBabe-puro for transient selection with puromycin to kill untrasfected cells.
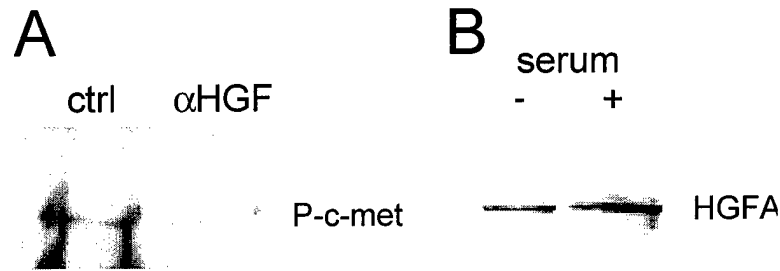
A   **B** serum
ctrl   αHGF   -   +

P-c-met   —— ——➤ HGFA

Figure 2: MPNST cells express HGFA and utilize HGF in an autocrine loop.
(A) ST8814 cells were grown in the presence of an anti-HGF neutralizing
antibody (αHGF) or an irrelevant control antibody ("ctrl") for 6 hrs. Lysates were
immunoprecipitated with a c-Met antibody then examined by western blot with a
phosphotyrosine antibody. Note that the level of phosphorylated c-Met ("P-c-
met") is dramatically reduced in cultures grown in the presence of the neutralizing
antibody. (B) Lysates of ST8814 cells grown in the presence and absence of fetal
bovine serum were analyzed for HGFA expression by western blotting. Note that
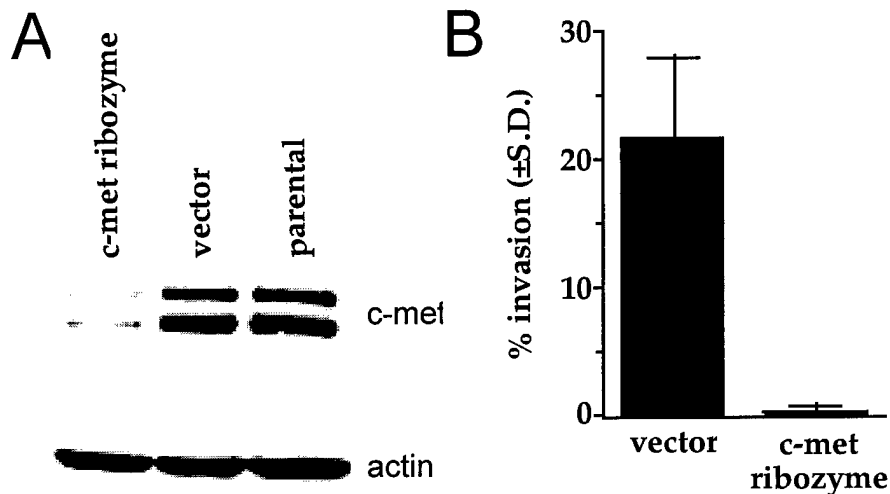ST8814 cells express HGFA, which is necessary for HGF processing.

A   **B**

c-met

actin

Figure 3: C-met mediates MPNST cell invasion. (A) Stable clones of ST8814
cells expressing a c-met ribozyme (1 of 3 is shown here) were analyzed by
Western blotting for c-met expression and expressed 70-80% less c-met compared
to stable clones expressing the ribozyme vector and parental ST8814 cells. (B)
The same clone shown in panel A is virtually non-invasive in a quantitative
Matrigel™ invasion assay compared to vector controls. Similar results were
obtained with the 2 other stable clones.

Prelimimary data described in the original proposal indicated that a dominant negative epidermal growth factor receptor (EGFR) construct could significantly reduce CD44 expression. This is interesting in light of recent findings indicating that MPNST cells have elevated EGFR expression (DeClue et al., 1999). To test whether elevated EGFR expression leads to aberrant CD44 expression in these cells through a Src-dependent mechanism, we utilized a dominant-negative EGFR construct that lacked the Src-association domain in the cytoplasmic tail but which still associated with other intracellular signaling molecules (Biscardi et al., 1999). We found that this construct also inhibited CD44 expression (Fig. 1b) consistent with Src activation being involved in EGFR-mediated activation of CD44 transcription.

Objective 2: Determine if CD44 contributes to MPNST cell invasion *in vitro* by influencing c-Met signaling

A major aim of this objective is to determine the contribution of the receptor tyrosine kinase c-Met and its ligand, hepatocyte growth factor (HGF), to MPNST invasion. The rationale for these experiments is that MPNST cells and tumor tissues express both c-Met and HGF (Rao et al., 1997), and recent data suggest that HGF signaling may depend on heparin sulfate modified forms of CD44 (van der Voort et al., 1999). We previously found that MPNST cells express these CD44 variants (Sherman et al., 1997). We have verified that MPNST cells express both HGF and c-Met, and found that an HGF-neutralizing antibody can significantly reduce c-Met phosphorylation in the ST8814 MPNST cell line (Fig. 2a). Furthermore, we found that MPNST cells express HGF-activating protein (HGFA), a proteolytic enzyme that is required for converting pre-HGF into mature, active HGF (Fig. 2b). These data demonstrate that MPNST cells signal in response to HGF in an autocrine manner.

We originally proposed using a dominant negative c-Met construct to inhibit constitutive c-Met activity in MPNST cells and to test if c-Met activity is required for MPNST cell invasion. However, we found that these constructs were not a reliable means of reducing c-Met phosporylation in MPNST and other cell lines (data not shown). As an alternative approach, we obtained ribozyme contructs to target both c-Met and HGF translation (Abounader et al., 1999) from Dr. John Laterra (The Johns Hopkins University School of Medicine, Baltimore, Maryland). We have now made stable ST8814 cell lines expressing the c-Met ribozyme and show that compared to stable lines expressing vector alone, clones of cells expressing the c-Met ribozyme have dramatically reduced levels of total c-Met protein (Fig. 3a). These clones are also significantly less invasive than control clones (Fig. 3b), consistent with the notion that the constitutive c-Met activity in MPNST cells promotes their metastatic behavior. We are presently generating additional ST8814 clones that express the HGF ribozyme, and plan to test both sets of clones in nude mouse tumorigenesis and metastasis assays.

Objective 3: Test if reducing CD44 expression inhibits MPNST growth or metastasis *in vivo*

Our preliminary data indicated that antisense CD44 oligonucleotides could transiently reduce CD44 expression and inhibit MPNST cell invasion *in vitro*. Our first goal in this objective was to generate an ecdysone-inducible antisense CD44 construct that could be used to reduce CD44 expression in MPNST cells. We have cloned this

construct and we are presently testing it in transient transfection assays before generating stable ST8814 clones for use in tumorigenesis studies in nude mice.

KEY RESEARCH ACCOMPLISHMENTS
- Demonstrated that elevated EGFR expression contributes to aberrant CD44 expression in MPNST cells through a Src-dependent mechanism
- Demonstrated that Src activity alone can abrogate aberrant CD44 expression
- Determined that HGF and c-Met co-expression by MPNST cells forms an active autocrine loop
- Determined that MPNST cells express HGFA
- Found that reduction of c-Met expression is sufficient to inhibit MPNST cell invasion *in vitro*

REPORTABLE OUTCOMES
A manuscript describing our findings on the regulation of CD44 expression by EGFR and Src, and the role played by CD44 in MPNST cell invasion is in preparation and should be submitted within one month. We also anticipate submitting a second manuscript within the next 6 months describing the interactions between CD44 components of the HGF-c-Met autocrine loop.

In addition to the manuscripts, the inducible antisense CD44 constructs are available to other investigators who are interested in this system.

CONCLUSIONS
Collectively, our findings to date indicate that aberrant CD44 expression contributes to the invasive properties of MPNST cells, possibly by facilitating c-Met activity. We further find that this abnormal CD44 expression is due to elevated EGFR expression that is linked to Src activation. Ongoing studies will determine the mechanisms by which CD44 influences c-Met activation, and whether HGF processing requires CD44. These data are significant in that they implicate aberrant Src and c-Met signaling in MPNST invasion and metastasis. The finding that HGFA is expressed by these cells also suggests that it, too, is a potential anti-metastasis therapeutic target.

REFERENCES
Abounader R, Ranganathan S, Lal B, Fielding K, Book A, Dietz H, Burger P, Laterra J. (1999). Reversion of human glioblastoma malignancy by U1 small nuclear RNA/ribozyme targeting of scatter factor/hepatocyte growth factor and c-met expression. *J. Natl. Cancer Inst.* 91:1548-56

Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, Parsons SJ. (1999). c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J. Biol. Chem.* 274:8335-43

DeClue JE, Heffelfinger S, Ling B, Li S, Rui W, Vass WC, Viskochil D, Ratner N. (1999). Epidermal Growth Factor Receptor Expression in Neurofibromatosis type 1 (NF1)-related Tumors and NF1 Animal Models. *J. Clinical Invest.* In press.

Lamb RF, Hennigan RF, Turnbull K, Katsanakis KD, MacKenzie ED, Birnie GD, Ozanne BW. (1997). AP-1-mediated invasion requires increased expression of the hyaluronan receptor CD44. *Mol Cell Biol.* 17:963-76

Naor D, Sionov RV, Ish-Shalom D. (1997). CD44: structure, function, and association with the malignant process. *Adv Cancer Res.* 71:241-319.

Rao UN, Sonmez-Alpan E, Michalopoulos GK. (1997). Hepatocyte growth factor and c-MET in benign and malignant peripheral nerve sheath tumors. *Hum. Pathol.* 28:1066-70

Sherman L, Jacoby LB, Lampe J, Pelton P, Aguzzi A, Herrlich P, Ponta H. (1997). CD44 expression is aberrant in benign Schwann cell tumors possessing mutations in the neurofibromatosis type 2, but not type 1, gene. *Cancer Res.* 157:4889-97

van der Voort R, Taher TE, Wielenga VJ, Spaargaren M, Prevo R, Smit L, David G, Hartmann G, Gherardi E, Pals ST. (1999). Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. *J. Biol. Chem.* 274:6499-506